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Reference Values for Apolipoprotein A-I and Apolipoprotein B in Serum Still Depend on Choice of Assay Techniques

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Summary: In a study with young children receiving growth hormone treatment, it was necessary to re-establish reference values for various blood components, e. g. apolipoproteins A-I and B. We find that considerable method-to-method variation still exists. We used the DuPont Dimension analyzer (D) and the Beckman Array analyzer (B) systems and a third procedure with Orion reagents and a Kone Analyzer (K). We investigated if assay results may be pooled or exchanged within our study. In 59 serum samples we measured apolipoprotein A-I and apolipoprotein B (n = 58) and calculated the orthogonal regression equations $y = a(S_a)\,x + b(S_b)$.

For apolipoprotein A-I the results are:

(I) B = 1.165 (0.065) D − 0.193 (0.077), S_{yx} = 0.055; with r = 0.922 and
(II) K = 0.831 (0.056) D − 0.190 (0.066), S_{yx} = 0.055; with r = 0.898.

For apolipoprotein B the equations are

(III) B = 1.586 (0.137) D − 0.246 (0.100), S_{yx} = 0.061; with r = 0.840 and
(IV) K = 0.869 (0.065) D + 0.251 (0.048), S_{yx} = 0.044: with r = 0.875.

According to *Passing & Bablok*, the slopes and intercept values are 1.093 and −0.126; 0.848 and 0.167; 1.500 and −0.185; 0.880 and 0.249. The overall impression is the same for both regression methods: comparability had not yet been achieved by early 1993, particularly not for apolipoprotein B, and reference values differ significantly depending on the selected methodology.

Introduction

Several reports in the literature have documented significant progress with regard to improving the analytical reliability of apolipoprotein analysis. The lack of inter laboratory comparability is notorious and still hampers the use of clinical chemical data severely. Activities undertaken at the Centers for Disease Control (CDC), Atlanta, USA (*G. R. Cooper* et al.) (1, 2) and at the Northwest Lipid Research Laboratories in Seattle, USA (*J. J. Albers, S. M. Marcovina* et al.) paved the way towards a coordinated project (3–5) with the International Federation of Clinical Chemistry (IFCC).

In parallel with a search for matrix compatible reference materials (6), the effect of lyophilisation was also studied (7, 8). It turned out that reference serum for apolipoprotein A-I could be manufactured and that standardizing the apolipoprotein B calibration, although it is a more difficult protein with respect to solubility, also has come within reach. Recently the results of the use of a common serum calibrator for apolipoprotein A-I determination have been published. The succesful transfer of analytical accuracy was demonstrated (9). The World Health Organization (WHO) has approved this material, SP1-01, as the WHO-IFCC International Reference Material for Apolipoprotein A-I.

As a result of the IFCC project, the usual situation of non-comparable assay data (10) may be expected to improve drastically. Very recently the succesful validation of the WHO-IFCC International Reference Material for Apolipoprotein B (SP3-07) was published as well (11).

In a study on reference values of various blood lipid fractions in young children, we analyzed the same set of serum samples in all three cooperating laboratories.

We will eventually compare and integrate these data with earlier studies on reference values in children.

Our long standing interest in lipid standardization activities led us to investigate the effect upon the reference ranges of the laboratory measurement (12, 13).

With regard to the measurement of apolipoprotein A-I and B, we hoped the results of at least two of our methods would be comparable, because the manufacturers of the reagents and calibrators (DuPont de Nemours (Nederland) BV and Beckman Instruments Nederland BV) had previously indicated to us that their assay systems could be considered "standardized" (apolipoprotein A-I as well as apolipoprotein B) as a result of their cooperation in the IFCC International Standardization of the immunochemical determination of apolipoprotein A-I and B. Beckman Instruments Inc. circulated a notification to this effect as early as June 10, 1991 (14). At the time we carried out our measurements no such information had been obtained from the third manufacturer (Orion).

Materials and Methods

Patients' blood samples

The study was approved by the Ethics Committee of the Rotterdam University Hospital and written informed consent was obtained from the parents of 59 healthy children aged 1.9–9.9 years, who came to the Sophia Childrens Hospital for minor surgery, e.g. a circumcision of the penis or excision of the tonsils. Children with any disorders involving lipid metabolism were excluded. Venous blood was collected in the fasting state immediately after the anaesthetics were given.

The blood was centrifuged after allowing to clot for an hour. The serum was collected, fresh-frozen and stored at −75 °C for a period of a few months. At the end of the collection period lipid and apolipoprotein determinations were carried out within a few hours after thawing the serum aliquots. These sera were analysed using all 3 methods in the same week.

Apolipoprotein determinations

Apolipoprotein A-I and B were measured by three different methods:

Immuno-nephelometry

In the immuno-nephelometric, kinetic technique of the University Hospital (for adults) Dijkzigt, we used the Array Protein System (Beckman Instruments Co, Brea, CA 92621-6209, USA). Antibodies raised against specific human apolipoproteins are brought into contact with the serum sample (40 µl). The antigen-antibody reaction gives rise to aggregates that scatter light. The peak rate signal of light scattering is converted to concentration units on the basis of calibration with Apolipoprotein Calibrator "Apolipoprotein Cal".

This single point procedure verifies, and if necessary corrects, the position of a pre-programmed standard curve. The product information sheet indicates that the calibration is traceable to "the proposed WHO International Reference Materials for Apolipoprotein A-I". The product information about the apolipoprotein B calibration makes no mention of calibration material.

Method precision with lyophilized commercial quality controls: Between-run CV, week-to-week during 23 consecutive weeks in the first half of 1993, for apolipoprotein A-I (at 1.23 g/l) is 4.2% and for apolipoprotein B (at 1.16 g/l) is 3.8%.

Turbidimetry (Dimension)

In the turbidimetric immuno-assay of the Sophia Childrens University Hospital we used the Dimension Analyser (DuPont Medical, Wilmington, Delaware, USA). Antibodies raised against human apolipoproteins are brought into contact with the serum sample (2 µl). The resulting immune-complexes give rise to turbidity in the assay cuvette. Photometric extinction readings are converted into concentrations on the basis of calibration with DuPont Apolipoprotein Calibrators. A five-point standard curve is applied.

Precision with commercial quality control sera of apolipoprotein A-I: At a low concentration (0.48 g/l) our between-run CV is 5.6% and at a high concentration (1.88 g/l) the CV is 4.5%.

Turbidimetry (Orion/Kone)

In the turbidimetric immuno-assay of the Department of Epidemiology we used the reagent kits of Orion Diagnostica (Espoo, Finland) and the Kone Specific Analyzer (Kone, Espoo, Finland).

The method is based on turbidimetric measurement at 340 nm of immunoprecipitation, enhanced by polyethylene glycol. Diluted antibody solution (150 µl) is added to a 15-fold serum dilution (30 µl) with buffer solution supplied with the kit. The increases in absorbance are recorded at a fixed time (30 minutes) after reagent mixing. A lyophilized calibrator is included in the kit and applied as a five-point standard curve.

According to the manufacturer, calibration values have been obtained by direct comparison with reference materials provided by CDC. Two other commercially available reference standards (Boehringer Mannheim, Mannheim, Germany and Immuno AG, Vienna, Austria) and Precinorm L (Boehringer Mannheim, Germany) served as controls.

Method characteristics (Orion): The Department of Epidemiology started semi-automated apolipoprotein analyses in 1988 as part of a population based survey (EPOZ study, (12)). In the second half of 1988, they also participated in an international survey (10) and five vials of lyophilized poolsera (high, medium, low) were analyzed in duplicate on five days by all participants. The results for apolipoprotein A-I were respectively (overall mean (n = 82), turbidimetry mean (n = 23), our result): 1.04, 0.98 and 0.94; 0.86, 0.81 and 0.84; 0.43, 0.40 and 0.40 g/l. The average CV was 6.1%.

For apolipoprotein B the results were: 1.04, 0.94 and 0.79; 0.84, 0.80 and 0.71; 0.46, 0.45 and 0.40 g/l.

The average CV was 5.6%.

Results

The apolipoprotein determinations yielded a total of 58–59 complete data sets from all three laboratories.

The Dimension analyzer was arbitrarily designated as "x" and the Array and Orion/Kone methods as "y" in both comparisons appearing below. Orthogonal regression analyses (*Deming & Morgan*, (15)) and regression calculations according to *Passing & Bablok* (16) gave the results listed in table 1.

We calculated reference ranges for apolipoprotein A-I and B with data obtained by each of our three methods as the mean ± 2 SD's, since medians hardly differed from the means, and we found the following ranges.

Apolipoprotein A-I reference ranges (g/l)

DuPont 0.80 − 1.56,
Beckman 0.76 − 1.60,
Orion/Kone 0.81 − 1.49.

Apolipoprotein B reference ranges (g/l)

DuPont 0.46 − 0.98,
Beckman 0.62 − 1.28,
Orion/Kone 0.53 − 1.11.

Tab. 1 Values from the regression equations $y = ax + b$ of method comparisons on apolipoprotein A-I and apolipoprotein B assays. Concentration units g/l.

A. x = DuPont Dimension and y = Beckman Array:

	Apolipoprotein A-I n = 59		Apolipoprotein B n = 58	
	Deming & Morgan	*Passing & Bablok*	*Deming & Morgan*	*Passing & Bablok*
r	0.922		0.840	
a	1.165	1.093	1.586	1.500
Sa	0.065		0.137	
b	−0.193	−0.126	−0.246	−0.185
Sb	0.077		0.100	
Syx	0.055		0.061	

B. x = DuPont Dimension and y = Orion/Kone

	Apolipoprotein A-I n = 59		Apolipoprotein B n = 58	
	Deming & Morgan	*Passing & Bablok*	*Deming & Morgan*	*Passing & Bablok*
r	0.898		0.875	
a	0.831	0.848	0.869	0.880
Sa	0.056		0.065	
b	0.190	0.167	0.251	0.249
Sb	0.066		0.048	
Syx	0.055		0.044	

Subscript:
The assays were compared by performing orthogonal (according to *Deming & Morgan*) as well as *Passing & Bablok* regression analyses, leading to equations $y = ax + b$; where a and b are the slope and y-intercept respectively − given with their standard deviations Sa and Sb − and r is the correlation coefficient.
Assay methods were (1) DuPont Dimension analyzer and (2) Beckman Array analyzer with their reagent systems and (3) Orion reagents on a Kone analyzer.

Discussion

For the evaluation of paediatric patients receiving growth hormone therapy, it was necessary to re-establish reference ranges for apolipoprotein A-I and B.

The three cooperating laboratories involved in this study use three different apolipoprotein assays. The measurement of serum apolipoproteins is usually connected with establishing coronary risk profiles and the need to standardize such measurements is well recognized. Studies in the eighties revealed that commercial calibrator materials were not commutable for the various immuno-precipitation methods as used by clinical laboratories (1). This causes in part significant inter-method assay variations. To enhance the accuracy of routine methods, certified international reference serum had to be developed which required a collaborative study of major manufacturers and leading research laboratories.

On the basis of (limited) information available to us from two of our manufacturers, we hoped to find reasonably comparable test results, although it may have been too early to expect such results to dissipate into daily practice. These manufacturers had participated in the IFCC Standardization Project for Measurements of Apolipoprotein A-I and B.

The outcome for apolipoprotein A-I was published in 1993 (9) and for apolipoprotein B more recently (11). The confirmatory method comparison for apolipoprotein A-I was performed in part 3 of the study after each single method had been calibrated on previously checked target values for the in-house materials. This check of target values occurred after the materials were assayed 4 times in 2 runs per day on each of 5 different days (n = 40) in parallel with the proposed WHO-IFCC reference material SP1-01. In order to confirm the outcome of the standardization of in-house standards, 50 human serum samples were analyzed in duplicate in 2 different runs. The results demonstrate that comparable apolipoprotein A-I results can be obtained by a variety of immunochemical methods through the use of certified reference materials. We would like to discuss this result in more detail.

Indeed the success is reflected by an overall inter-assay CV of less than 5% and by an average absolute bias of no more than 2.8%. Still it is interesting to take a closer look at some of the participants (9) since they seem to deviate quite far from the ideal regression equation. Table 3 in the paper in question (9) shows a few participants in the IFCC study with slopes deviating significantly from 1.0. To estimate the effect of this on reference ranges, we calculated the effect upon a hypothesized accurate reference range of 0.70−1.50 g/l. The chosen four laboratory results from l. c. (9) are:

$y = 0.79\ x + 0.25,$
$y = 0.82\ x + 0.21,$
$y = 1.16\ x - 0.21,$
and $y = 1.20\ x - 0.22.$

An accurate reference range of 0.70−1.50 g/l, recalculated for use in these four laboratories, would lead to:

0.80 − 1.44,
0.78 − 1.44,
0.60 − 1.53,
and 0.62 − 1.58 g/l.

The differences among these calculated reference ranges are not small enough to be neglected and they arise in well standardized centres.

Our own results do show intermethod differences of the same order of magnitude. Our upper ranges for apolipoprotein A-I vary from 1.49 to 1.60 g/l.

Although the examples above concern only 4 out of 29 methods for apolipoprotein A-I, we feel that 4/29 are no rare exceptions and they underline the fact that the transfer of accuracy to general clinical laboratories is no easy matter.

For the standardization of apolipoprotein B, there now is a WHO-IFCC International Reference Material available as a well-stabilized liquid serum product: SP3-07.

Table 3 in the paper (11) shows to what extent uniformity among methods was obtained. The 25 analytical systems show regression equations between

$y = 0.83\ x + 0.15$
and $y = 1.14\ x - 0.13.$

For these two cases, the above discussion on apolipoprotein A-I applies as well, in that a manufacturer must not use these data as the basis for producing secondary calibrators. Both examples fall within the arbitrary criteria for being considered standardized for apolipoprotein B because the overall imprecision is $< 5\%$ and overall bias is $< 6\%$. Still, a low slope combined with a positive y-intercept, or vice versa, a high slope combined with a negative y-intercept, although both lead to the same average apolipoprotein values, result in very different reference ranges.

Clearly these comparisons demonstrate that reference ranges differ too much to be interchangeable. We expect that the manufacturers in the IFCC studies will re-adjust their method calibrations to finally obtain slopes within 5% of unity and y-intercepts within 0.1 g/l of zero. This does not at all question the fact that great progress will be made in harmonizing lipoprotein measurements when using the International Reference Materials.

In paediatric laboratory medicine, there is a frequent lack of reference values due to the enormous difficulties in obtaining proper samples in high enough numbers (17). This matter is a subject for further study.

In conclusion, the comparability among our three apolipoprotein A-I as well as among our three apolipoprotein B methods is not good enough to pool the analytical data nor to use common reference values.

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